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Granzyme A Initiates an Alternative Pathway for Granule-Mediated Apoptosis

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Summary

Granzyme (gzm) B–deficient cytotoxic lymphocytes (CTL) have a severe defect in the rapid induction of target cell apoptosis that is almost completely corrected by prolonged incubation of the CTL effectors and their targets. We show in this report that perforin-dependent, gzmB-independent cytotoxicity is caused by gzmA (or tightly linked genes). CTL deficient for gzmA and gzmB retain normal perforin function, but these CTL have a cytotoxic defect in vivo that is as severe as perforin-deficient CTL. Collectively, these results suggest that perforin provides target cell access and/or trafficking signals for the gzms, and that the gzms themselves deliver the lethal hits. The gzmA pathway appears to function independently from gzmB and may therefore provide a critical "back-up" system when gzmB is inhibited in the target cell.

Introduction

Cytotoxic lymphocytes are armed with two major contact-dependent mechanisms for inducing apoptosis in their targets. The first is the granule exocytosis pathway, which is predominantly utilized by $CD8^+$ cytotoxic T lymphocytes (CTL), natural killer cells (NK), and lymphokine-activated killer cells (LAK). After these cytotoxic lymphocytes conjugate with target cells, their cytotoxic granules vectorally migrate toward the site of contact, followed by secretion of granule contents into the space between the effector and the target cell (Henkart, 1994). In the classic model, granule-associated perforin creates a pore in the target cell membrane through which other granule constituents pass; the most abundant of the granule proteins are the granzymes (gzms), which are neutral serine proteases that have the ability to induce apoptosis upon target cell entry (Henkart, 1994; Shresta et al., 1998). The other major contact-mediated cytotoxic mechanism is the Fas system, which is predominantly used by $CD4^+$ T cells (Rouvier et al., 1993). After T cell receptor engagement, the Fas ligand gene is activated, and the newly synthesized Fas ligand is displayed on the surface of CTL, where it can interact with Fas receptor on the surface of the target cell. Fas receptor engagement leads to activation of the caspases, which ultimately cleave substrates that are critical for the induction of apoptosis (Enari et al., 1996, 1998; Liu et al., 1997).

The precise role of perforin in the granule exocytosis pathway is not completely understood. Perforin is processed and polymerizes upon exposure to calcium. Polyperforin is capable of inducing damage to membranes that causes the release of proteins from target cells (e.g., ^{51}Cr release). Although this membrane damage can induce target cell necrosis, perforin alone is not sufficient to cause the apoptosis of nucleated cells in vitro (Duke et al., 1989; Shi et al., 1992a, 1992b; Shiver et al., 1992). Recently, several investigators have questioned the role of polyperforin molecules as "entry sites" for gzms (Shi et al., 1996; Jans et al., 1996; Pinkoski et al., 1998; Froelich et al., 1998). They have instead suggested that gzms may enter target cells in a perforin-independent manner, where they reside in a protected cytoplasmic compartment; perforin then acts to release the gzms from this protected compartment, allowing them to translocate to the nucleus, where they cleave critical death substrates. Regardless, both hypotheses suggest that perforin is required for the gzms to gain access to their apoptotic substrates in the target cell.

A variety of in vitro studies have suggested that granzymes A and B (gzmA and and gzmB) are important for the induction of target cell apoptosis (Pasternak and Eisen, 1985; Hudig et al., 1991; Shi et al., 1992a, 1992b; Nakajima et al., 1995). GzmB is thought to be a rapidly acting apoptotic enzyme, while gzmA is thought to be slow acting (Shi et al., 1992a, 1992b). CTL derived from mice deficient for gzmB have a severe defect in the rapid induction of apoptosis, but this defect is almost completely corrected by prolonged incubation of the effectors and targets (Heusel et al., 1994; Shresta et al., 1995). CTL derived from mice deficient for gzmA have no detectable defect in cytotoxicity in vitro (Ebnet et al., 1995; Shresta et al., 1997a), but they do display a defect in the clearance of the orthopoxvirus Ectromelia (Mullbacher et al., 1996).

In this report, we characterize mice that are deficient for both gzmA and gzmB. As previously reported (Pham et al., 1996), the gzmB loss-of-function mutation used here causes loss-of-function for gzms C, D, and F, which lie just downstream from gzmB in a multi-gene cluster; this "cluster knockout" probably is caused by the retained PGK-*neo* cassette in the gzmB gene. The gzmA and -B cluster-deficient mice have a defect in both the rapid and slow induction of cytotoxicity that is similar to perforin-deficient mice. However, these mice have normal quantities of fully functional perforin, suggesting that the membrane damage caused by perforin is insufficient to cause target cell death in vitro or in vivo. Collectively, our results suggest that perforin facilitates gzm entry and/or trafficking, and that gzmB rapidly delivers the lethal hit to the target cell. In the absence of functional gzmB, gzmA plays a critical role in the induction

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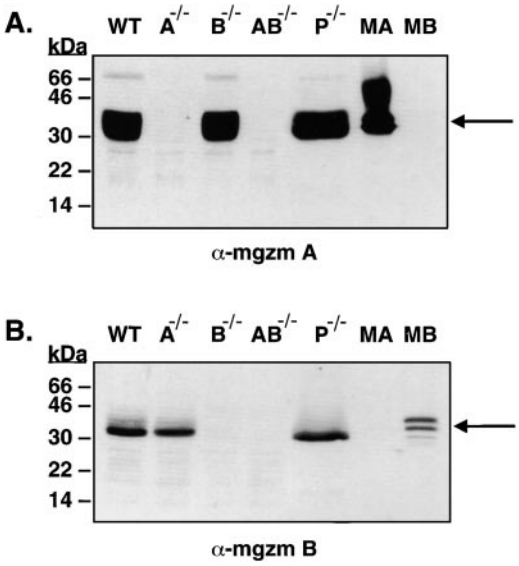

Figure 1. Lack of GzmA or GzmB Protein in Cytotoxic Lymphocytes Derived from GzmA$^{-/-}$ × GzmB$^{-/-}$ Mice

Whole-cell extracts were prepared from 1 × 10^6 LAK cells derived from wild-type (WT), gzmA$^{-/-}$ (A$^{-/-}$), gzmB$^{-/-}$ (B$^{-/-}$), gzmA$^{-/-}$ × gzmB$^{-/-}$ (AB$^{-/-}$), and perforin$^{-/-}$ (P$^{-/-}$) mice. Recombinant gzmA (MA) (0.2 μg) and gzmB (MB) (0.1 μg) were prepared from supernatants of *P. pastoris* cultures. Proteins were analyzed via Western blotting using a previously described (Shresta et al., 1995) rabbit anti-murine gzmB antiserum (B) and a rabbit anti-mouse gzmA antiserum (A). Samples in both panels were prepared using denaturing conditions; therefore, both gzmA and gzmB monomers recognized by these antisera migrated at approximately 30 kDa. The position of gzmA is shown by the arrow in (A), and the position of gzmB is shown by the arrow in (B). The heterogenous bands represent different glycosylated forms of these enzymes.

of apoptosis. The gzmA pathway may therefore be important for CTL-mediated killing of target cells that contain inhibitors of gzmB.

Results

Characterization of GzmA$^{-/-}$ × GzmB$^{-/-}$ Mice

In our previous studies, cytotoxic lymphocytes derived from H-2^b mice (C57BL/6 × 129/SvJ) carrying a homozygous null mutation in the gzmA gene or gzmB gene were shown to lack gzmA or gzmB mRNAs, respectively (Heusel et al., 1994; Shresta et al., 1997a). Further studies demonstrated that effector cells from gzmA$^{-/-}$ mice did not show any reduction in the level of gzmK mRNA (Shresta et al., 1997a), whereas LAK cells from gzmB$^{-/-}$ mice demonstrated markedly decreased expression of downstream gzmB-cluster genes C through G, presumably due to a "neighborhood knockout" effect of the PGK-*neo* cassette retained in the mutant alleles (Pham et al., 1996). In the present study, we rederived these gzmB$^{-/-}$ and gzmA$^{-/-}$ mice into the 129/SvJ strain (H-2^b) and mated them with each other to obtain doubly heterozygous mutants. Southern analysis of tail DNA derived from the progeny of doubly heterozygous matings revealed an expected number of doubly homozygous mice (data not shown); these mice have normal development and fertility and are physically indistinguishable from their wild-type counterparts.

Flow cytometric analyses of cells derived from the thymus, lymph node, and spleen revealed normal hematopoiesis and lymphopoiesis in the gzmA$^{-/-}$ × gzmB$^{-/-}$ mice (data not shown). Splenocytes from these mice (H-2^b) were stimulated with allogeneic BALB/c splenocytes (H-2^d) in a primary, one-way mixed lymphocyte reaction (MLR) to generate allo-specific (H-2^b anti-H-2^d) CTL or with high-dose IL-2 to generate LAK cells. As expected, Western analysis using specific anti-gzmA or anti-gzmB antisera revealed a complete absence of gzmA and gzmB proteins in both MLR-CTL (data not shown) and LAK cells (Figure 1) derived from the doubly mutant mice.

GzmA$^{-/-}$ × GzmB$^{-/-}$ Cytotoxic Lymphocytes Have a Normal Ability to Proliferate and Become Activated In Vitro

We detected no difference in the number of MLR-CTL and LAK cells that could be generated from wild-type or gzmA$^{-/-}$ × gzmB$^{-/-}$ mice, indicating that these splenocytes can proliferate normally in response to allogeneic or IL-2 stimuli in vitro. Moreover, flow cytometric analyses demonstrated nearly equivalent percentages of CD3$^+$, CD4$^+$, and CD8$^+$ cells in MLR cultures derived from wild-type, gzmA$^{-/-}$ × gzmB$^{-/-}$, and perforin$^{-/-}$ mice (Figure 2A), which were used for comparisons in functional studies (discussed below). Additional flow cytometric studies revealed similar percentages of CD3$^+$, CD4$^+$, CD8$^+$, CD16$^+$, and NK1.1$^+$ cells in LAK populations from 129/SvJ wild-type versus gzmA$^{-/-}$ × gzmB$^{-/-}$ mice, whereas the proportion of NK1.1$^+$ cells was considerably higher in LAK cultures from perforin$^{-/-}$ mice, which were created in a mixed (C57BL/6 × 129/SvJ) strain (Figure 2B); this result is expected, since NK cells derived from the C57BL/6 strain express NK1.1, while 129/SvJ NK cells do not (Sentman et al., 1989). Regardless, wild-type and gzmA$^{-/-}$ × gzmB$^{-/-}$ effectors both expand in cultures in a similar fashion.

GzmA$^{-/-}$ × GzmB$^{-/-}$ Cytotoxic Lymphocytes Retain an Intact Perforin Pathway for Inducing Target Cell Membrane Damage

We examined the ability of gzmA$^{-/-}$ × gzmB$^{-/-}$ effectors to trigger membrane damage via perforin molecules: we focused on LAK cells, since they contain more perforin than MLR-CTL. As shown in Figure 3A, a Western analysis of LAK extracts revealed equivalent amounts of perforin (with the same electrophoretic mobility) in wild-type and gzmA$^{-/-}$ × gzmB$^{-/-}$ samples, further confirming the activation status of these cells, since perforin expression is restricted to activated CTL. We then tested the membranolytic activity of these extracts using hemolytic assays in which the amount of hemoglobin released from sheep red blood cells is measured spectrophotometrically. As shown in Figure 3B, perforin$^{-/-}$ LAK cell extracts have no ability to disrupt sheep red blood cell membranes, demonstrating that this assay is perforin dependent. In contrast, wild-type and gzmA$^{-/-}$ × gzmB$^{-/-}$ LAK cell extracts demonstrate the same dose-dependent ability to cause sheep red cell membrane damage.

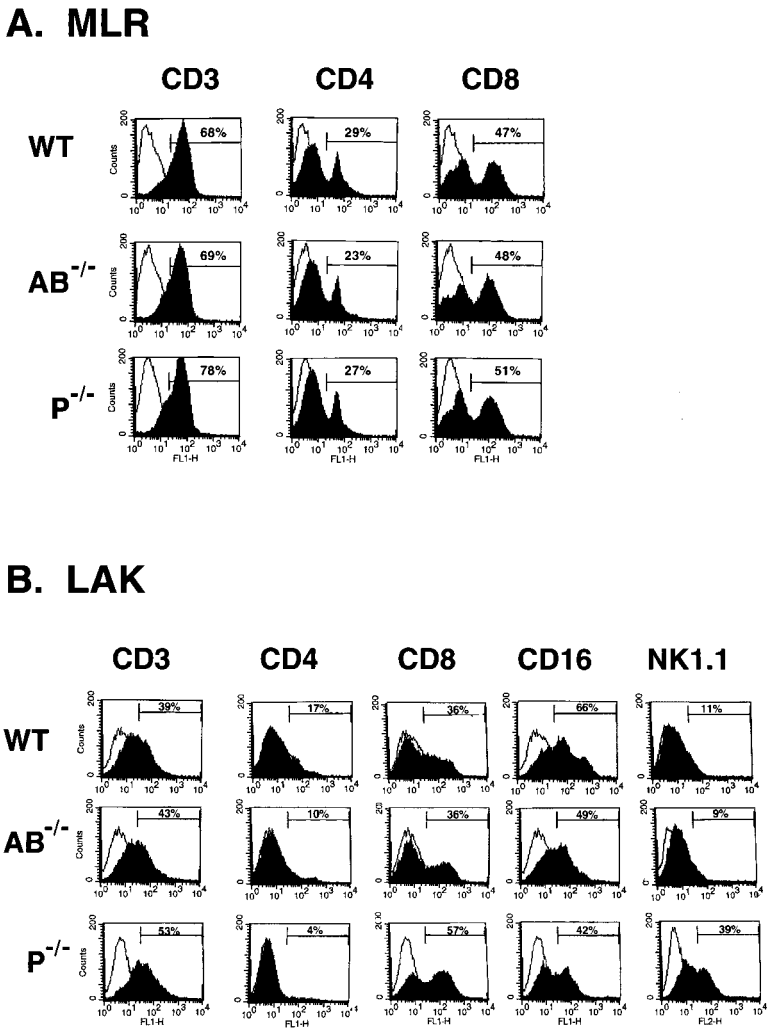

Figure 2. Normal Proliferation and Activation of Cytotoxic Lymphocytes Derived from GzmA$^{-/-}$ × GzmB$^{-/-}$ Splenocytes

Day 5 MLR cells (A) and high-dose IL-2-stimulated LAK cells (panel B) derived from wild-type (WT), gzmA$^{-/-}$ × gzmB$^{-/-}$ (AB$^{-/-}$), and perforin$^{-/-}$ (P$^{-/-}$) mice were analyzed by flow cytometry. The expression profiles of cells stained with FITC-labeled monoclonal antibodies specific for mouse CD3, CD4, CD8, CD16, PE-labeled NK1.1 (filled histograms) were overlaid with background fluorescence of cells stained with isotype control antibodies (open histograms). The percentages of cells within the gated windows was similar for all mice.

This result indicates that gzmA$^{-/-}$ × gzmB$^{-/-}$ LAK cells contain normal amounts of fully functional perforin.

GzmA$^{-/-}$ × GzmB$^{-/-}$ Cytotoxic Lymphocytes Have a Severe Defect in Their Ability to Induce Target Cell DNA Fragmentation after Prolonged Incubation with Allogeneic Target Cells

We examined the function of gzmA$^{-/-}$ × gzmB$^{-/-}$ effector cells using standard cytotoxicity assays, in which ^{51}Cr or ^{125}IUdR release was measured as a function of increasing E:T (effector:target) ratios against a fixed number of radiolabeled target cells; the effectors and targets were coincubated for 2 hr ("early") or 8 hr ("late"). We conducted these experiments using MLR-CTL instead of LAK cells for two reasons. First, we were able to test MLR-CTL against three different allogeneic target cell lines (TA3, P815, and YAC1), whereas we have only one reliable LAK-sensitive target cell line (YAC1). Second, as shown in Figure 2B, the NK1.1^{+} LAK cells from C57BL/6 × 129/SvJ perforin$^{-/-}$ mice are probably not appropriate controls for the NK1.1^{-} LAK cells derived from 129/SvJ wild-type or gzmA$^{-/-}$ × gzmB$^{-/-}$ mice. MLR cultures from all three types of mice contained relatively equal proportions of appropriate T cell subsets (Figure 2A), allowing us to directly compare their cytotoxic activities.

The cytotoxic activity of MLR-CTL against allogeneic TA3 (H-2^{d}) cells is shown in Figure 4. After 2 hr of incubation, perforin-deficient CTL induce no ^{51}Cr release from the target cells; gzmA$^{-/-}$ × gzmB$^{-/-}$ CTL mediate less ^{51}Cr release than wild-type CTL, but this defect varies among experiments for reasons that we do not yet understand (Heusel et al., 1994). After 8 hr of incubation, both wild-type and gzmA$^{-/-}$ × gzmB$^{-/-}$ CTL reproducibly induce equivalent ^{51}Cr release from their targets, while perforin$^{-/-}$ CTL remain defective. As expected, MLR-CTL derived from all three types of mice caused no ^{51}Cr (or ^{125}IUdR release) from syngeneic EL4 (H-2^{b}) target cells (data not shown). Therefore, both the ^{51}Cr release and hemolytic assays suggest that the gzmA$^{-/-}$ × gzmB$^{-/-}$ CTL are equipped with functional perforin.

The ^{125}IUdR release assays (right panels in Figure 4) demonstrate that gzmA$^{-/-}$ × gzmB$^{-/-}$ CTL are very similar to perforin$^{-/-}$ CTL in their inability to induce target cell DNA fragmentation. GzmA$^{-/-}$ × gzmB$^{-/-}$ CTL completely lack the ability to cause ^{125}IUdR release from target cells after 2 hr of incubation. This defect is entirely due to the gzmB mutation (Heusel et al., 1994). Per-

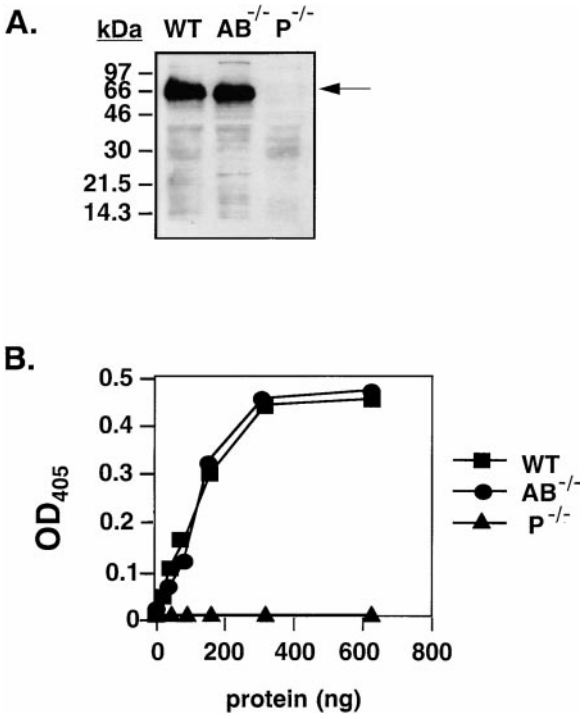

Figure 3. Normal Expression and Activity of Perforin in GzmA$^{-/-}$ × GzmB$^{-/-}$ LAK Cells

(A) LAK cell extracts (50 μg) derived from wild-type (WT), gzmA$^{-/-}$ × gzmB$^{-/-}$ (AB$^{-/-}$), and perforin$^{-/-}$ (P$^{-/-}$) mice were analyzed by Western blotting. The rat monoclonal antibody directed against mouse perforin detected similar amounts of the 60 kDa perforin protein in WT and AB$^{-/-}$ samples. The position of correctly processed perforin is shown by the arrow. The absence of perforin in the P$^{-/-}$ lane confirmed the specificity of this antibody.

(B) Wild-type (WT), gzmA$^{-/-}$ × gzmB$^{-/-}$ (AB$^{-/-}$), and perforin$^{-/-}$ (P$^{-/-}$) LAK cell extracts were assayed for hemolytic activity against sheep red blood cell membranes. The amount of hemoglobin released into the red cell supernatants was spectrophotometrically measured at 405 nM. OD_{405} values (y-axis) were plotted against increasing amounts of LAK cell extracts (x-axis). Similar results were obtained in four independent experiments.

forin$^{-/-}$ CTL are also completely defective for the early induction of ^{125}IUdR release, presumably because perforin is required to deliver gzmB to its intracellular targets. After 8 hr of incubation, the gzmA$^{-/-}$ × gzmB$^{-/-}$ and perforin$^{-/-}$ CTL have the same severe defect in the induction of ^{125}IUdR release from target cells. This result has been reproduced using two additional allogeneic target cell lines (P815 and YAC1; data not shown). Importantly, we have previously shown that neither gzmA$^{-/-}$ nor gzmB$^{-/-}$ CTL have substantial defects in the induction of ^{125}IUdR release after 8 hr of incubation with their targets (Heusel et al., 1994; Shresta et al., 1997a, 1997b). This result suggests that gzmA (or a tightly linked gene) is responsible for the target cell DNA fragmentation caused by gzmB$^{-/-}$ CTL after prolonged incubation with their targets.

Recombinant GzmB Partially Reconstitutes the Ability of GzmA$^{-/-}$ × GzmB$^{-/-}$ Cytotoxic Lymphocytes to Induce Target Cell DNA Fragmentation In Vitro

Since both the gzmA and gzmB mutant loci retain the PGK-*neo* cassette, the observed phenotype may be influenced by the downregulation of nearby genes due to "neighborhood" effects. Although the expression patterns of the known neighboring genes in both the gzmA and gzmB loci have already been reported, possible effects of other unidentified genes in the neighborhood cannot be ruled out, since the gzmA and gzmB clusters have not yet been fully characterized. Therefore, to determine whether gzmA and gzmB themselves were responsible for the defects observed in the gzmA$^{-/-}$ × gzmB$^{-/-}$ effectors, we developed recombinant gzms for use in ^{125}IUdR release assays to reconstitute the missing enzymes in these cells (Pham et al., 1998).

Figure 5 depicts the results of reconstitution assays using LAK cells incubated for 2 hr with YAC1 targets at a fixed E:T ratio of 30:1 (E = 3 × 10^5 cells; T = 10^4 cells). We previously demonstrated that the specific activity of recombinant murine gzmB is at least as great as that of native gzmB purified from wild-type LAK cells (Pham et al., 1998). Similarly, equal amounts of recombinant gzmA and native gzmA from wild-type LAK cells had equivalent BLT-esterase activity (data not shown). We performed Western analyses of whole-cell extracts prepared from known numbers of wild-type LAK cells and known amounts of the recombinant gzms; these blots revealed the relative amounts of recombinant enzyme added with respect to the native enzyme present in a fixed dose of wild-type LAK cells (Figure 5A). Densitometric analysis revealed that 3 × 10^5 wild-type LAK cells contained approximately 0.13 μg of gzmB and 0.24 μg of gzmA. These data suggest that the gzms comprise several percent of the wet weight of LAK cells, consistent with previous reports (Masson and Tschopp, 1987; Poe et al., 1991). The recombinant gzms were used at amounts ranging from 0.5 μg to 2.0 μg per 100 μl reaction; these doses do not alter the normal ability of wild-type LAK cells to induce ^{125}IUdR release from their targets (Figure 5B).

Perforin-deficient LAK cells are completely defective in their ability to induce ^{125}IUdR release with or without added gzms (Figure 5B). Recombinant gzmB, but not gzmA, partially restores the ability of gzmA$^{-/-}$ × gzmB$^{-/-}$ LAK cells to mediate ^{125}IUdR release in a dose-dependent manner (left and middle panels in Figure 5B). The effect of gzmB is specifically due to the active enzyme, since equal amounts of an attenuated Ser_{183}Ala mutant form of recombinant gzmB did not induce ^{125}IUdR release (data not shown). Therefore, these results confirm that gzmB itself is at least partially responsible for the induction of target cell DNA fragmentation and that gzmB activity in target cells requires perforin. The incomplete correction of target cell ^{125}IUdR release by gzmB may be due to the requirement for other tightly linked gzms in the cluster (e.g., gzms C, D, and/or F) or to an insufficient effective concentration of gzmB; we could not study the effect of gzmB at larger doses, since these doses reduced ^{125}IUdR release caused by wild-type effectors.

GzmA did not induce ^{125}IUdR release at any concentration tested. The combination of both gzmA and gzmB at equimolar concentrations had an effect similar to that of gzmB alone (right panel in Figure 5B). At present, the role of gzmA for the induction of apoptosis after prolonged incubation cannot be biochemically confirmed, since the target cells undergo spontaneous apoptosis in the serum-free buffer system required to support gzm activity for 8 hr of incubation (data not shown).

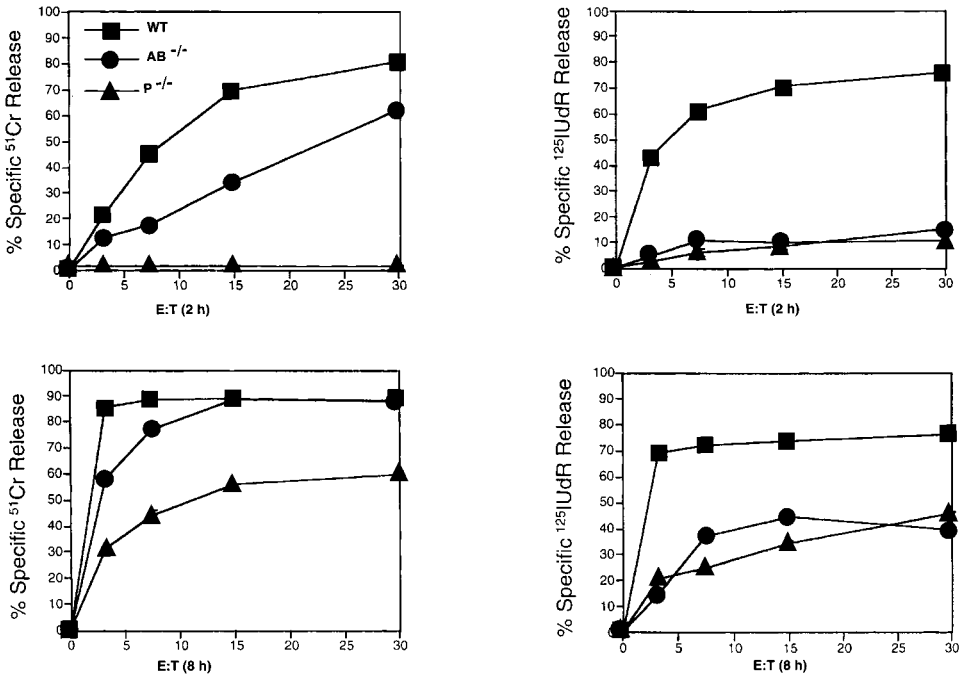

Figure 4. Cytotoxic Activity of GzmA$^{-/-}$ × GzmB$^{-/-}$ MLR Cells

In vitro cytotoxicity of allospecific (H-2^b anti-H-2^d) MLR cells from wild-type (WT), gzmA$^{-/-}$ × gzmB$^{-/-}$ (AB$^{-/-}$), and perforin$^{-/-}$ (P$^{-/-}$) mice was tested against the allogeneic (H-2^d) TA3 target cells at increasing E:T ratios (3.75:1 to 30:1; T = 10^4 cells) after 2 hr (top panels) and 8 hr (bottom panels) of incubation of effector with target cells. Data are presented as mean ± SEM of duplicate samples. The SEMs for most of the data points are very small and are contained within the symbols. This experiment is representative of ten experiments with similar results.

GzmA$^{-/-}$ × GzmB$^{-/-}$ and Perforin$^{-/-}$ CD8$^+$ CTL Have Similar Defects in an Acute Graft versus Host Disease Model

To evaluate the physiological significance of the in vitro cytotoxic defects observed in gzmA$^{-/-}$ × gzmB$^{-/-}$ CTL, we used an acute GVHD model (Sprent et al., 1986; Graubert et al., 1996, 1997) to examine the in vivo cytotoxicity of CD8$^+$ T cells. In this model, the recipients are (B6.C-H-2^{bm1} × 129/SvJ) F1 mice, which share all major histocompatibility loci with 129/SvJ gzmA$^{-/-}$ × gzmB$^{-/-}$ mice, except for a mutant H-2^{bm1} (class I) allele and minor histocompatibility antigens; the mutant bm1 allele creates a strong, class I–restricted allogeneic signal. These recipients were lethally irradiated and then rescued with an infusion of syngeneic marrow combined with CD4-depleted, CD8$^+$ T cells obtained from the mesenteric lymph nodes of sex-matched littermate wild-type mice, gzmB$^{-/-}$ mice, gzmA$^{-/-}$ mice, gzmA$^{-/-}$ × gzmB$^{-/-}$ mice, or perforin$^{-/-}$ mice. Following recognition of the bm1 signal on the recipient cells, wild-type T cells become activated and attack the bm1$^+$ hematopoietic cells in the graft, leading to death of the recipients from marrow failure; in contrast, CD8$^+$ T cells with defective cytotoxicity do not destroy the graft, and the recipients can survive with normal hematopoiesis.

As previously reported (Graubert et al., 1996, 1997), all mock-transplanted recipients died within 2 weeks due to marrow aplasia (data not shown), while all mice transplanted with marrow only and no T cells survived (Figure 6A). T cells derived from wild-type or gzmA$^{-/-}$ mice caused the death of all recipients within 30 days posttransplant (Figure 6A). In our previous studies, we demonstrated that gzmB$^{-/-}$ CD8$^+$ T cells allowed a significant number (20%–30%) of the recipients to survive, while perforin$^{-/-}$ CD8$^+$ T cells permitted 90% survival in this model (Figure 6A; Graubert et al., 1997). In the present study involving the gzmA$^{-/-}$ × gzmB$^{-/-}$ mice, wild-type CD8$^+$ T cells caused the death of all recipients within 55 days of the transplant, while perforin$^{-/-}$ CD8$^+$ T cells again allowed >75% of the recipients to survive ($P < 0.001$) (Figure 6B). Similarly, CD8$^+$ T cells from gzmA$^{-/-}$ × gzmB$^{-/-}$ mice also allowed >80% of recipients to survive ($P < 0.001$) and were therefore indistinguishable from T cells derived from perforin$^{-/-}$ mice ($P = 0.32$) (Figure 6B). The median survival time of the recipients reconstituted with wild-type CD8$^+$ T cells was 38 days in this study versus 24 days in past studies, perhaps because of the lower CD8$^+$ T cell dose used in this study, improved survival in our new barrier facility, or strain differences between the previous experiments (C57BL/6 × 129/SvJ) and this one (129/SvJ). Each set of experiments was internally controlled in that the T cell dose resulted in 100% mortality for the wild-type group and >90% survival for the perforin$^{-/-}$ group. This allows a meaningful comparison of the results for the gzm-deficient effectors between experiments.

Discussion

In this report, we describe the phenotype of gzmA$^{-/-}$ × gzmB$^{-/-}$ mice made in the 129/SvJ (H-2^b) background. The evaluation of CTL derived from these mice has revealed that the perforin-dependent cytotoxicity of gzmB$^{-/-}$ mice can be accounted for by gzmA or a tightly linked gene. The relevance of gzmA for CTL function is also emphasized by the in vivo studies described in this report, which demonstrate that gzmA plays a large role in

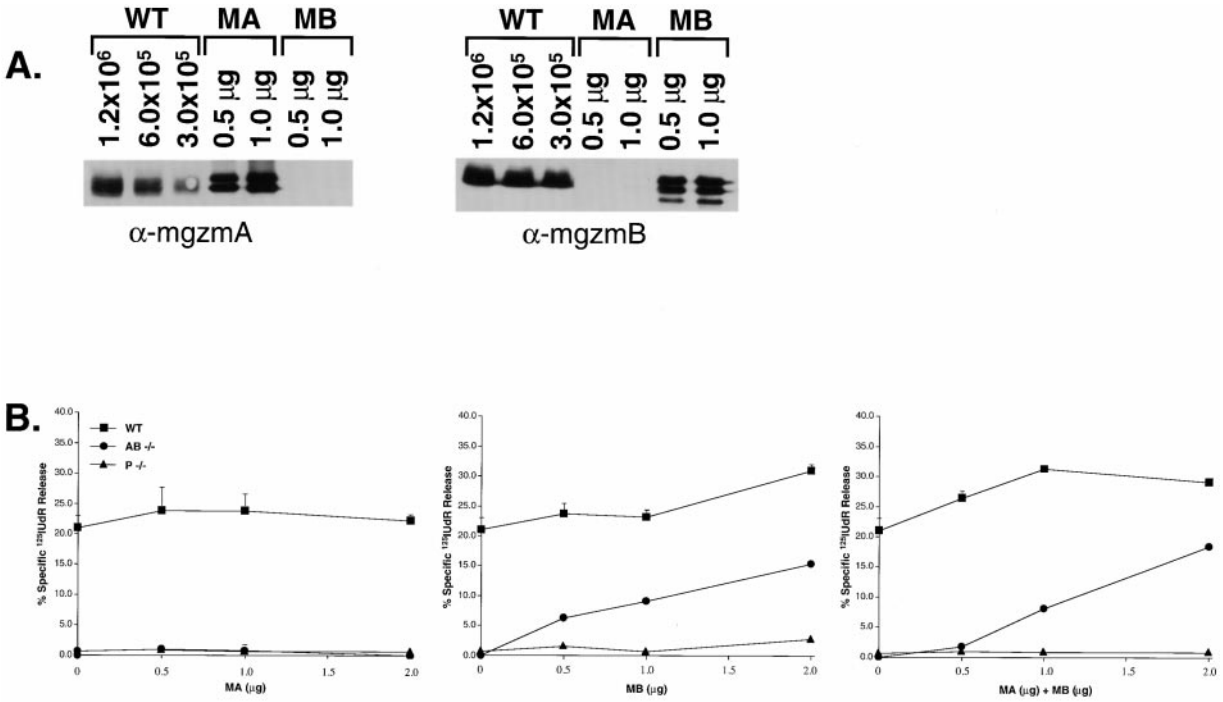

Figure 5. Partial Reconstitution of the Early Target Cell DNA Fragmentation Pathway in the GzmA$^{-/-}$ × GzmB Cluster$^{-/-}$ LAK Cells by Recombinant GzmB

(A) Total cell extracts from fixed numbers of wild-type LAK cells (WT) and known amounts of recombinant gzmA (MA) and gzmB (MB) were analyzed by Western blotting using rabbit antisera against mouse gzmA (left panel) and mouse gzmB (right panel).

(B) A 2 hr ^{125}IUdR release assay was performed using 3.0 × 10^5 LAK cells derived from wild-type (WT), gzmA$^{-/-}$ × gzmB$^{-/-}$ (AB$^{-/-}$), and perforin$^{-/-}$ (P$^{-/-}$) mice and 10^4 YAC1 targets (E:T ratio = 30:1; incubation time = 2 hr). Recombinant mature gzmA (MA) and/or mature gzmB (MB) were added into each mixture of effector and target cells. Three different amounts (0.5, 1.0, and 2.0 μg) of each recombinant enzyme were used. Data are represented as mean ± SEM of duplicate samples. Similar results were obtained in five separate experiments.

the overall cytotoxic potential of CD8$^+$ effectors. We have also shown here that effector cells armed with perforin but not with gzmA or gzmB cluster enzymes are able to induce target cell membrane damage in in vitro settings (frequently referred to as "necrosis"), but this membrane damage does not lead to target cell death in an in vivo model system. These results strongly suggest that the membrane damage induced by perforin does not directly cause target cell death; rather, perforin facilitates the action of the death effector molecules, the gzms. Our results also suggest that the gzmA and gzmB loci contain most, if not all, of the death effector machinery delivered to target cells via perforin.

The results presented in this paper confirm and extend the results reported by Simon and colleagues (1997), who also evaluated the cytotoxicity of lymphocytes derived from gzmA × gzmB deficient animals. The gzmA$^{-/-}$ mice in their study were made by inserting a PGK-*neo* cassette into exon 4 of the gzmA gene; the genetic background of the gzmA$^{-/-}$ mice was C57BL/6. The gzmB$^{-/-}$ mice were obtained from our laboratory and were created in a mixed 129/Sv × C57BL/6 background. The gzmA mutation used in this study was made by deleting exon 2 of the gzmA gene, and the mutation was made in 129/SvJ mice (Shresta et al., 1997a). We rederived the gzmB mutation in 129/SvJ mice for this study so that the genetic background of all of the mice is identical. Regardless, the results of the in vitro cytotoxicity assays between the two studies are similar.

In the present study, we characterized the abundance and function of perforin in gzmA$^{-/-}$ × gzmB$^{-/-}$ mice. Cytotoxic lymphocytes derived from these mice have normal amounts of perforin of the correct size (Uellner et al., 1997), and lysates made from these cells have normal levels of perforin activity in a sheep red blood cell lysis assay that specifically measures perforin activity. Similarly, we have shown that gzmA$^{-/-}$ × gzmB$^{-/-}$ effectors induce ^{51}Cr release from their target cells; ^{51}Cr release measures protein leakage caused by membrane damage and is an assay that can be affected by multiple pathways. However, early ^{51}Cr release is due to perforin-mediated events in the target cell (Kagi et al., 1994a; Kojima et al., 1994; Lowin et al., 1994; Walsh et al., 1994), while at later time points, ^{51}Cr release is caused not only by perforin-induced damage but also by membrane damage induced by internal disintegration of the cell from the Fas pathway (Kagi et al., 1994b; Lowin et al., 1994; Braun et al., 1996; Shresta et al., 1997b). Both the in vitro and in vivo studies described here show that gzmA$^{-/-}$ × gzmB$^{-/-}$ CTL have a severe defect in their ability to cause target cell death—a defect that is similar in magnitude to the defect observed in perforin-deficient CTL. These results confirm the observation that perforin alone cannot kill target cells and suggest that the membrane damage caused by perforin can be repaired by the target cells if the gzms are not delivered (Duke et al., 1989; Shi et al., 1992a, 1992b; Shiver et al., 1992).

No consistent cytotoxic defect has been detected in

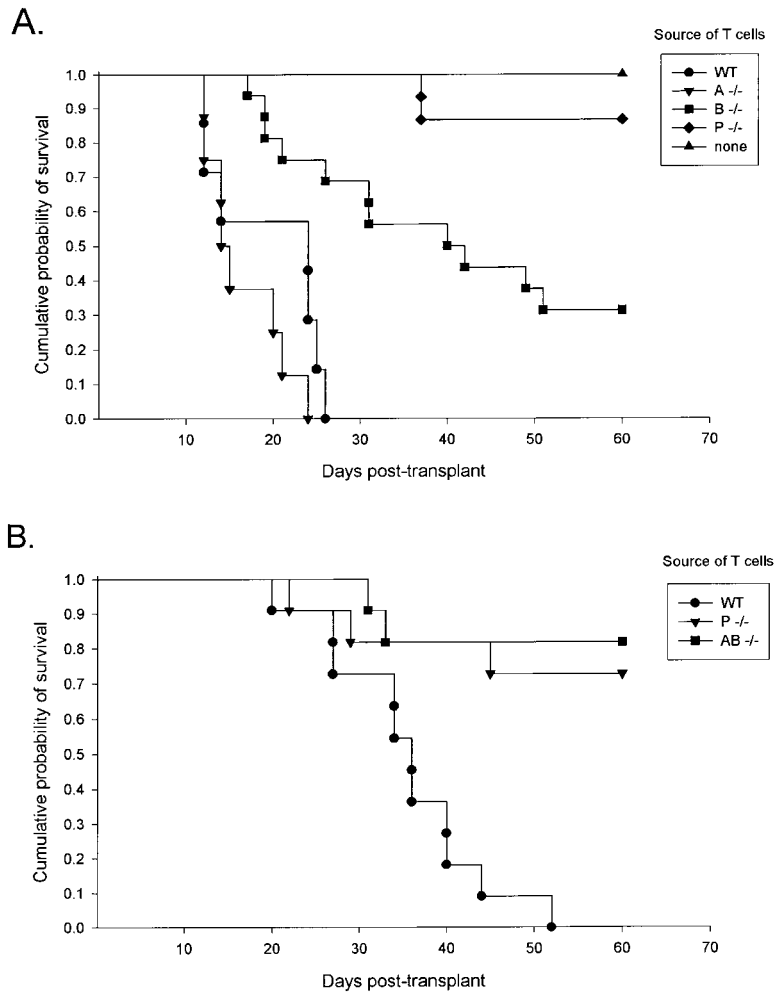

Figure 6. The Phenotypes of GzmA$^{-/-}$ × GzmB$^{-/-}$ and Perforin$^{-/-}$ CD8$^+$ Effector Cells Are Similar in an Acute Graft versus Host Disease Model

(A) Phenotype of CD8$^+$ T cell effectors from mice deficient for gzmA or gzmB. Bm1 × 129/SvJ recipient mice were lethally irradiated and were then reconstituted with 2×10^6 syngeneic bone marrow cells mixed with 1×10^6 class I–mismatched CD8$^+$ T cells from wild-type (WT), perforin$^{-/-}$ (P$^{-/-}$), gzmA$^{-/-}$ (A$^{-/-}$), or gzmB cluster$^{-/-}$ (B$^{-/-}$) mice. The Kaplan-Meier plot demonstrates that survival from graft-versus-host disease was similar when gzmA$^{-/-}$ CD8$^+$ T cells (n = 8) were infused, as compared to wild-type CD8$^+$ T cells (n = 7). Survival was significantly prolonged when gzmB cluster$^{-/-}$ CD8$^+$ T cells were used (n = 16) ($p < 0.01$). When perforin$^{-/-}$ CD8$^+$ T cells were used (n = 15), survival was indistinguishable from control transplants using syngeneic marrow without T cells (n = 4). Data for this curve were pooled from three independent experiments, each of which had a similar outcome. The results for the perforin$^{-/-}$ and gzmB$^{-/-}$ mice have been previously published (Graubert et al., 1997) and are shown for comparison.

(B) Phenotype of CD8$^+$ T cell effectors from gzmA$^{-/-}$ × gzmB$^{-/-}$ versus perforin$^{-/-}$ mice. Class I–mismatched transplants were performed as described in (A), except that 1.5×10^5 CD8$^+$ cells were infused. The Kaplan-Meier plot shows that ~75% of recipients reconstituted with CD8$^+$ T cells from either gzmA$^{-/-}$ × gzmB$^{-/-}$ (n = 11) or perforin$^{-/-}$ (n = 11) mice survived. As expected, all recipients with wild-type CD8$^+$ T cell donors (n = 11) died. Data for this curve were pooled from three independent experiments, each of which had a similar outcome.

vitro with gzmA-deficient mice generated by either our group (Shresta et al., 1997a) or by Simon and colleagues (Ebnet et al., 1995). However, the role of gzmA for cytotoxic lymphocyte-mediated function is clearly demonstrated in the gzmA × gzmB deficient mice. The previous studies of Shi and colleagues (1992b) and Shiver and colleagues (1992) strongly suggested that gzmA was important for CTL-mediated cytotoxicity, and Nakajima et al. (1995) suggested that it could somehow synergize with gzmB to effect apoptotic death. Our results extend those landmark studies. GzmA is extremely important for CD8$^+$ and LAK-mediated cytotoxicity in vitro and in vivo, but the effect of gzmA deficiency is masked by an intact gzmB pathway in the gzmA$^{-/-}$ mice. Although gzmB$^{-/-}$ CTL have a profound defect in the rapid induction of apoptosis in their targets, this defect is almost completely corrected by prolonged incubation of the effectors and targets (Heusel et al., 1994; Shresta et al., 1995a). Some of this "correction" is provided by the Fas system, and the rest is perforin dependent (Shresta et al., 1997b). The perforin-dependent component is clearly due to gzmA (or a tightly linked gene) both in vitro and in vivo. These results suggest that gzmA plays an essential role in CTL cytotoxicity *only* when effectors lack gzmB or when target cells contain inhibitors of gzmB, as discussed below.

We and others have recently been able to successfully express fully active recombinant gzms using different approaches (Beresford et al., 1997; Harris et al., 1998; Pham et al., 1998; Xia et al., 1998). The reconstitution assay described in this report relies upon the ability of the recombinant gzms to enter the target cell via a perforin-dependent mechanism. Large absolute excesses of recombinant gzmB can partially, but not completely, correct the cytotoxic defect observed with gzmA$^{-/-}$ × gzmB$^{-/-}$ effector cells; this effect is dependent on the proteolytic activity of gzmB, since an equal amount of an attenuated version of recombinant gzmB was inactive in this assay. This result clearly proves that at least part of the phenotype of the gzmB mutation is due to deficiency of gzmB itself. However, the partial reconstitution leaves open the possibility that the expression of additional genes within the cluster (e.g., gzms C, D, or F) are required for the full reconstitution of early cytotoxicity. To address this question, we have developed mice that contain a null mutation of gzmB only by removing the PGK-*neo* cassette from the mutant locus using Cre-recombinase mediated excision. The preliminary analysis of these mice suggests that gzmB itself is responsible for the rapid induction of apoptosis in vitro (D. A. T. and T. J. L., unpublished data).

Alternatively, the partial reconstitution may reflect the

inefficiency of the assay system; we are adding ~5- to 20-fold molar excesses of recombinant gzms compared to the amount present within the defined number of wild-type effectors used in the assay. However, the "effective concentration" of the recombinant gzms may be quite low; the effector cell gzms are highly concentrated within the granules of these cells and are delivered precisely to the target cells after a tight junction is established. The extracellular, recombinant gzms used in this assay may enter target cells through pores in the target cell membrane created by perforin or may enter via a nonperforin-dependent mechanism. This delivery mechanism may be very inefficient and may explain why we cannot completely correct the cytotoxic defect with exogenous enzyme.

We were not able to assess the role of gzmA in the reconstitution assay with prolonged incubations, because the target cells underwent spontaneous apoptosis in the serum-free buffer that was required to maintain gzm activity in these assays. However, we did learn that gzmA cannot substitute for gzmB—nor synergize with it—in the 2 hr reconstitution assay; these results strongly suggest that gzmA must induce apoptosis via a non-overlapping mechanism from gzmB. Gzm B is an aspase (Odake et al., 1991; Thornberry et al., 1997; Harris et al., 1998) that is known to cleave and activate several procaspases upon target cell entry (Darmon et al., 1995, 1996; Martin et al., 1996; Quan et al., 1996; Talanian et al., 1997); however, the requirement of caspase activation for the induction of target cell apoptosis remains highly controversial (Sarin et al., 1997; Talanian et al., 1997; Andrade et al., 1998). One biochemical substrate for gzmA (a tryptase) has been identified (pHAP-2) (Beresford et al., 1997), but the physiologic substrates of gzmA in the apoptosis pathway are completely unknown. Furthermore, we do not know whether gzmA and gzmB cause apoptosis by activating the same distal apoptotic substrates.

Collectively, these results suggest a model for how the perforin-gzm system functions (see Figure 7). In this model, perforin is responsible for gaining entry into the target and delivering the molecules that cause the lethal hits. GzmB can be likened to a grenade that detonates almost immediately in the target. GzmA behaves like a bomb with a delayed fuse. If gzmB can attack its critical apoptotic substrates, the target cell is quickly killed; the gzmA pathway cannot be detected under these circumstances, because it is destroyed in the dying cell. However, if gzmB fails to be delivered or is somehow inactivated in the target cell, the long-fused gzmA bomb can still ultimately kill the target. This model suggests that gzms A and B do *not* synergize with each other in the classic sense but provide two distinct, independent pathways that can each cause the apoptotic death of the cell. This model does not explain why gzmB acts rapidly and why gzmA acts slowly. Gzm A may initiate apoptosis by activating an alternate pathway that has many protease components, akin to the complement pathway or the coagulation cascade. Alternatively, this enzyme may directly cleave critical apoptotic target proteins but at a slow rate. Regardless, the gzmA pathway is functional in the absence of gzmB, and it therefore represents an alternate pathway for the induction of apoptosis.

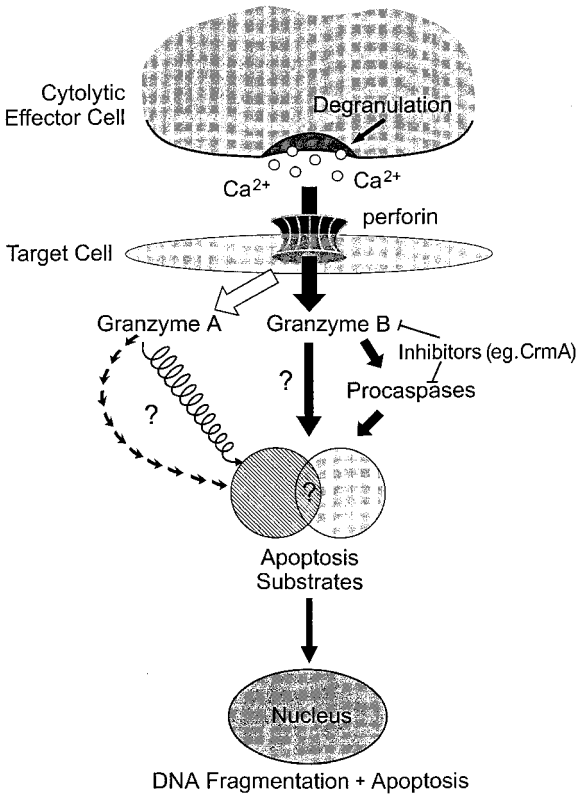

Figure 7. A Model for the GzmA and GzmB Pathways

This model suggests that the gzmA pathway is independent from gzmB, even though these pathways could potentially converge on common substrates (indicated by overlapping circles). The gzmA pathway induces target cell apoptosis slowly, either because this enzyme is kinetically slow (indicated by the corkscrew) or because it acts through multiple substrates before the apoptotic substrates are reached (indicated by the multiple arrow pathway). Regardless, the gzmA pathway cannot be detected unless the gzmB pathway is inactivated, either by a loss-of-function mutation in the effector cell (e.g., gzmB$^{-/-}$ mice) or by inhibiting the activity of gzmB in the target cell. As an example, the poxvirus crmA gene encodes a serine proteinase inhibitor (serpin) that interacts with and inhibits the activities of apoptotic caspases and gzmB. Under these circumstances, gzmA may still be able to kill the target cell via an independent apoptotic mechanism (see Discussion for details).

Why do CTL granules contain two distinct enzymes for inducing apoptotic death in target cells? Our results suggest that the gzmA pathway may have evolved as a back-up pathway for gzmB in virus-infected cells (Smyth and Trapani, 1998). Pickup and colleagues first identified a cowpox gene that encodes a serpin (serine proteinase inhibitor) known as the cytokine response modifier A (CrmA) protein (Ray et al., 1992). CrmA binds to and inactivates caspases and also gzmB (Quan et al., 1995; Zhou et al., 1997); overexpression of CrmA in target cells can inhibit the rapid induction of apoptosis by CTL (Tewari et al., 1995). Recently, Mullbacher and colleagues demonstrated that the orthopoxvirus Ectromelia is cleared slowly in gzmA-deficient mice (Mullbacher et al., 1996). Ectromelia contains a CrmA homolog (87% identical to CrmA), and therefore cells infected with this virus are probably resistant to gzmB-induced apoptosis. Under normal circumstances, the

gzmA pathway is required to clear the virus, since gzmB is inhibited; if gzmA is not present, the virus persists. These results predict that any virus that expresses a functional inhibitor of gzmB might be cleared more slowly in gzmA-deficient mice. Experiments to confirm this hypothesis are in progress.

There are other circumstances where the gzmA pathway may be important because of inhibition of the gzmB pathway. For example, parainfluenza virus type 3 infection can apparently reduce the abundance of gzmB mRNA in CTL (Sieg et al., 1995), which would reduce the delivery of gzmB to the target cell. Alternatively, gzmB could potentially be inhibited in target cells by cellular serpins (Sun et al., 1996; Hampson et al., 1997; Bird et al., 1998) or inhibitor of apoptosis proteins (IAPs) related to the baculovirus p35 IAP (Hay et al., 1995; Rothe et al., 1995; Xue and Horvitz, 1995; Hawkins et al., 1996; Deveraux et al., 1997; Irmler et al., 1997). IAP expression has been detected in tumors and tumorigenic cell lines (Irmler et al., 1997); inappropriate expression of IAPs could protect these cells from apoptotic stimuli delivered by a variety of apoptotic stimuli, including gzmB. If gzmA does not activate the same substrates targeted by the caspases and gzmB, its activity would not be inhibited by any of these molecules.

In summary, these results establish an important role for gzmA in cytotoxic effector cells and demonstrate that perforin-induced membrane damage does not cause irreversible changes in target cells that cause their death. The irreversible changes are caused by gzms A or B (or tightly linked genes), which probably induce target cell death via independent apoptotic pathways; we do not know whether these pathways converge on the same apoptotic substrate or different ones. Future studies will be directed toward the identification and comparison of the key apoptotic pathways activated by these very different enzymes.

Experimental Procedures

Creation of GzmA$^{-/-}$ × GzmB Cluster$^{-/-}$ Mice

To obtain doubly deficient mice in a pure 129/SvJ (H-2^b) background, 129/SvJ females were first mated with gzmA-chimeric chimeric males (obtained via injecting targeted RW4 embryonic stem [ES] cells into C57BL/6 blastocysts) or with gzmB cluster–chimeric males (obtained via injecting targeted D3 ES cells into C57BL/6 blastocysts). The resulting single heterozygotes were then intercrossed to produce mice deficient for both gzmA and gzmB cluster proteases. All mutant mice used for the experiments in this report (including perforin-deficient mice in a mixed C57BL/6 × 129/SvJ background) were identified by Southern blotting analysis of restriction enzyme–treated tail DNA and were 6–12 weeks of age.

Production of Active Recombinant Murine GzmA

The cDNA encoding the mature form of mouse gzmA was PCR-amplified and subcloned into the pCR 2.1 vector (Invitrogen) using the forward (5′-CTCGAGAAAAGAATCATTGGAGGAGACACGGTTG TTCCTCACTCAAGACCG-3′) and reverse (5′-TTACACAGAACCC TTC-3′) primers, which add a XhoI site and the α-factor signal peptide sequence of *Pichia pastoris* at the 5′ end of the cDNA. The XhoI–EcoRI insert was then cloned into the *P. pastoris* expression vector pPIC9 (Invitrogen). After completely confirming the insert sequence in pPIC9, this vector was electroporated into GS115, and the resulting His$^+$ clones were selected and induced with methanol exactly as described for the production of recombinant mouse gzmB. Supernatants of the induced His$^+$ clones were tested for tryptase activity on a daily basis, and a clone with a high level of tryptase activity after 4 days of induction was chosen to be expanded into 2L cultures. The day 4 supernatant of 2L cultures was purified using an Accell Plus cation exchange column (Waters), as previously described. The purity of recombinant gzmA, which eluted at 0.70–0.75 M NaCl, was confirmed by silver staining (ICN Biomedicals). The expected monomeric (28–32 kDa) and homodimeric (60–70 kDa) forms of gzmA were detected in reducing SDS-PAGE and nonreducing gels, respectively.

Generation of Anti-Mouse GzmA Antiserum

Polyclonal rabbit antiserum against mouse gzmA was obtained by immunizing rabbits (EL Labs) with recombinant gzmA protein that was purified as described above. The specificity of the antiserum was confirmed by comparing Western analysis of wild-type versus gzmA$^{-/-}$ effector cell lysates and recombinant mouse gzmA versus recombinant mouse gzmB protein.

Western Blot Analyses

Effector cell lysates, which were prepared via a freeze-thaw procedure (see below in Hemolytic Assays), and recombinant gzms were analyzed using a standard Western blotting technique, and signals were detected by enhanced chemiluminescense via horseradish peroxidase linked anti-rabbit or anti-rat Ig secondary antibodies at a 1:5000 dilution (Amersham Life Science). The primary antibodies used were rabbit anti-mouse gzmA antiserum (described above) at a 1:1000 dilution, rabbit anti-mouse gzmB antiserum at a 1:1000 dilution, and rat anti-mouse perforin monoclonal antibody (clone P1–8) (Kamiya Biomedical Company) at 5 μg/ml.

Tryptase Activity Assays

The tryptase activity of recombinant and native gzmA was determined using N-α-benzylcarbonyl-L-lysine thiobenzyl ester (BLT) as a substrate, as previously described (Shresta et al., 1997a).

Effector Cells, Target Cells, and In Vitro Lytic Assays

The production of effector cells, labeling of target cells, and performance of in vitro lytic assays was previously described (Heusel et al., 1994; Shresta et al., 1995).

Hemolytic Assays

Sheep red blood cells (Clonetics) were washed and resuspended at 3×10^7 cell/ml in HBSS, 2 mg/ml BSA, 0.01 M HEPES (pH 7.4), and 2.5 mM $CaCl_2$. Freeze-thaw extracts of LAK cells were prepared by washing cells twice in PBS, followed by resuspension at 10^7 cells/100 μl in PBS/1 mM EDTA, frozen in a dry ice/ethanol bath for 10 min, and thawed in a 4°C bath. The resulting cell extracts were briefly sonicated, spun in a microcentrifuge to remove cellular debris, and the total protein concentration in the cleared lysate was quantitated using a Bio-Rad protein assay (Bio-Rad). Varying concentrations of the cell lysate (in a final volume of 50 μl PBS/1 mM EDTA) were mixed with 150 μl of the sheep red blood cell suspension in a 96-well plate, and the plate was incubated for 20 min at 37°C. Following centrifugation of the plate, the amount of hemoglobin present in the supernatants was measured at A_{405} nM using a microplate reader (Molecular Devices).

In Vitro Reconstitution Assays

Target cells were labeled with ^{125}IUdR, and effector cells were harvested exactly as described for standard lytic assays (above). Next, fetal calf serum was removed by washing both target and effector cells in Hank's balanced salt solution (HBSS) three times, followed by final resuspension in H5 medium (HBSS supplemented with 4 mg/ml fatty acid free BSA, 2 mM $CaCl_2$, and 10 mM HEPES [pH 7.4]). Target cells (10^4 in 50 μl H5), effector cells (3×10^5 in 50 μl H5), and 100 μl of recombinant gzms in HE buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, and 1 mM EGTA) were mixed together and incubated at 37°C. All recombinant gzms used were produced in *P. pastoris*. The recombinant enzymes were purified on Accell plus cation exchange resin (Waters), dialyzed at 4°C against 100 volumes of HE buffer, and quantitated using the BCA Protein Assay Reagent (Pierce). Procedures employed for standard lytic assays were followed to determine percent specific ^{125}IUdR release. Target cells incubated in HE buffer alone served as spontaneous controls. The

addition of recombinant gzms at concentrations ranging from 2.5 ng/μl to 10.0 ng/μl had no effect on percent specific release values measured in the spontaneous controls.

In Vivo Cytotoxicity Assays

$CD8^+$ T cells present in mesenteric lymph nodes of wild-type, $gzmA^{-/-}$, $gzmB^{-/-}$, $gzmA^{-/-} \times gzmB^{-/-}$, or $perforin^{-/-}$ mice were purified as described (Graubert et al., 1997). Bone marrow cells (2×10^6 per recipient mouse) from (B6.C-H-2^{bm1} × 129/SvJ) F1 hosts were prepared and infused intravenously with 1.5×10^5 $CD8^+$ T cells into irradiated hosts as described (Graubert et al., 1996).

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